Inhaled Particulate Matter Causes Expression of Nuclear Factor (NF)-κB–Related Genes and Oxidant-Dependent NF-κB Activation *In Vitro*

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High levels of ambient air pollution are associated with exacerbation of asthma and respiratory morbidity, yet little is known concerning the mechanisms of inflammation and toxicity by components of inhaled particulate matter (PM). Brief inhalation of $PM_{2.5}$ (particles of an aerodynamic diameter of < 2.5 microns) ($300~\mu g/m^3$ air for 6 h followed by a period of 24 h in clean air) by either C3H/HeJ or C57/BL6 mice caused significant ($P \leqslant 0.05$) increases in steady-state messenger RNA (mRNA) levels of a number of nuclear factor (NF)-κB–associated and/or –regulated genes, including tumor necrosis factor-α and -β, interleukin-6, interferon-γ, and transforming growth factor-β. Lung mRNA levels of lymphotoxin-β and macrophage migration inhibitory factor were unchanged. In murine C10 alveolar cells and an NF-κB–luciferase reporter cell line, exposure to $PM_{2.5}$ at noncytotoxic concentrations resulted in increases in transcriptional activation of NF-κB–dependent gene expression which were inhibited in the presence of catalase. Early and persistent increases in intracellular oxidants, as measured by flow cytometry and cell imaging using the oxidant probe 2′-7′-dichlorofluoroscin diacetate, were observed in epithelial cells exposed to $PM_{2.5}$ and ultrafine carbon black particles. Studies here are the first to show NF-κB–related inflammatory and cytokine gene expression after inhalation of $PM_{2.5}$ and oxidant-dependent induction of NF-κB activity by $PM_{2.5}$ in pulmonary epithelial cells.

Ambient particulate matter (PM) is a complex mixture of chemicals and particles that may be compositionally diverse depending on geography and season (1). A number of studies suggest that brief, high-level exposures to PM may exacerbate cardiopulmonary disorders and unscheduled admissions to hospitals (1, 2). Increases in air pollution have also been associated with aggravation of asthma as measured by decreased lung function values, shortness of breath, and emergency department visits (3). In addition, long-term exposures to PM have been linked to possible increases in lung cancer risk, chronic respiratory disease, and death rates (4, 5). The interpretation of these data has been

questioned (6), primarily because of the lack of mechanistic, toxicologic, or epidemiologic studies necessary to establish cause-and-effect relationships between exposure to PM and disease outcomes. For example, inhalation studies in rodents have failed to demonstrate adverse pulmonary pathology after acute exposures to PM (7, 8), and more sensitive biomarkers and methods of analysis may be needed to detect subtle PM-induced effects in the lung.

In the present work we hypothesized that acute exposures to PM elicit increased expression of key genes involved in inflammation, a process associated with and/or integral to the initiation of a number of lung diseases. We used a multiprobe ribonuclease protection assay (RPA) as a sensitive tool to examine expression of cytokine genes in the lungs of two different strains of mice after a brief period (6 h) of inhalation of $PM_{2.5}$ (particles of an aerodynamic diameter of < 2.5 microns). To understand the mechanisms of PM-induced gene expression, we used an alveolar type II epithelial cell line (C10) (9) as a primary target of inhaled PM which is deposited in the alveolar duct region and peripheral lung after inhalation. Alveolar type II epithelial cells produce a battery of chemokines and cytokines important in inflammation and cell proliferation. Moreover, their injury and aberrant function are also linked to the development of interstitial lung diseases (reviewed in 10). Because inhalation of $PM_{2.5}$ caused increased expression of genes regulated by and/or associated with the transcription factor nuclear factor (NF)-κB, we also examined whether NF-κB activity and NF-κB–dependent gene expression were increased in C10 cells exposed to $PM_{2.5}$.

NF-κB is a transcription factor that is activated by a number of oxidant stresses. Thus, an important aspect of our work was to determine whether $PM_{2.5}$ caused oxidant-dependent activation of NF-κB and production of oxidants by pulmonary epithelial cells. Lastly, because PM is a complex mixture of organic and inorganic substances including coarse (aerodynamic diameter = 2.5–10 microns), fine (aerodynamic diameter = 0.1–2.5 microns) and ultrafine (aerodynamic diameter = < 0.1 microns) particulates, we also examined well-characterized samples of $PM_{2.5}$, ultrafine carbon black (uCB) (average diameter = 0.05 microns), and glass beads (GB) (diameter of 1 to 4 microns), a nonpathogenic control particle, to determine the properties of PM important in activation of NF-κB and oxidant production. Our studies indicate that the ultrafine particulate component of $PM_{2.5}$ plays an important role in oxidant production and NF-κB activation.

Materials and Methods

Inhalation Experiments

Male C3H/HeJ and C57/BL6 mice (22 to 34 g, The Jackson Laboratory, Bar Harbor, ME) were exposed to $PM_{2.5}$ at the NYU Medical Center in Manhattan, NY, using nose-only exposures. Two inbred mouse strains were examined comparatively here because of their differential inflammatory responses to ozone (11). In these studies, ozone-exposed C57/BL6 mice exhibited increased numbers of neutrophils in bronchoalveolar lavage fluid (BALF) in comparison with the C3H/HeJ strain. A centrifugal concentrator (12) was used to concentrate entrained urban $PM_{2.5}$ approximately 10-fold, yielding a gravimetric mass concentration of 250 μg/m³ air. Mice were exposed to $PM_{2.5}$ for 6 h, and groups (*n* = 2–3/strain/time point) were killed using a lethal intraperitoneal injection of pentobarbital at 0 and 24 h after exposure. These time points were chosen because they reflected maximum increases in neutrophils and protein, respectively, in lavaged samples of ozone-exposed mice (11). Sham control mice were treated identically but exposed to filtered clean air. At both time points, the lungs of sham and $PM_{2.5}$-exposed mice were lavaged using a saline solution to determine total cell numbers and differential cell counts, and the lung tissue was immediately frozen in liquid nitrogen for isolation of RNA (13).

RPA

Total RNA was isolated from frozen lavaged lung tissues as described previously (13), quantitated by absorbance at 260 nm, and analyzed using an RPA system and a multiprobe template set (mCK-3b) for tumor necrosis factor (TNF)-α, TNF-β, lymphotoxin (LT)-β, interleukin (IL)-6, interferon (IFN)-γ, transforming growth factor (TGF)-β1, TGF-β2, TGF-β3, and macrophage migration inhibitory factor (MIF) (RiboQuant; PharMingen, San Diego, CA). The template set also included mouse ribosomal protein (L32) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal controls. The RNA duplexes were isolated by extraction/precipitation, dissolved in 5 μl gel loading buffer, and electrophoresed in standard 5% acrylamide/urea sequencing gels. After gels were dried, autoradiograms were developed and quantitated using a Bio-Rad PhosphorImager (Bio-Rad, Hercules, CA). Results were normalized to expression of the internal control, L32.

Preparation and Characterization of $PM_{2.5}$ Samples and Other Particulates for *In Vitro* Studies

Vermont (VT) $PM_{2.5}$ samples were collected on Teflon filters from the Burlington and Waterbury, VT, monitoring stations using a Wedding collection apparatus. Within 24 h after collection, VT $PM_{2.5}$ was removed from filters using sonication (four times for 30 s each in 1 ml of pyrogen-free water). Preparations were then aliquoted, lyophilized, and stored at −80°C before use, i.e., fresh $PM_{2.5}$. uCB particles were obtained from Monarch 880CB (Cabot, Billerica, MA). The particle size distribution and elemental analysis of each sample were then determined using transmission electron microscopy and energy-dispersive X-ray mass analysis as detailed previously (14). GB (1 to 4 microns diameter) were obtained from Particle Information Services, Inc., Kingston, WA.

Cell Cultures and Addition of Particulates

The C10 cell line is a nontumorigenic murine alveolar type II epithelial cell line originally cloned from the NAL 1A alveolar type II epithelial cell line (9). The line was isolated from adult mice and maintains a characteristic epithelial morphology including surface microvilli, desmosomes, and lamellar bodies. C10 cells were maintained and passaged in CMRL 1066 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics. At confluence, cells were switched to 0.5% FBS-containing medium for 24 h before addition of VT $PM_{2.5}$ samples or other particulates. All particles were weighed and suspended at 1 mg/ml in Hanks' balanced salt solution (HBSS) (Life Technologies) before addition at final nontoxic concentrations to cell cultures. At 1 h, particles had precipitated onto cells as determined by phase and scanning electron microscopy. The cytotoxicity of particles over a 48-h period was assessed in initial experiments using a cell impermeant fluorescent dye, Sytox (Molecular Probes, Eugene, OR), and flow cytometry. These studies showed that $PM_{2.5}$ and uCB were not cytotoxic at concentrations ≤ 10 μg/cm² dish. Assays were performed on C10 cells after addition of particulates for 1, 2, 4, and 8 h on the basis of previous characterization of NF-κB activation by asbestos in mesothelial and epithelial cells (15, 16).

Preparation of Nuclear Extracts and Electrophoretic Gel Mobility Shift Assays

Nuclear extracts of sham or particle-treated C10 cells were prepared as described previously (14). The amount of protein in each sample was determined using the Bio-Rad protein assay (Bio-Rad). A total of 3 μg of each sample was then mixed with 10 μl 2× binding buffer (80 mM *N*-2-hydroxyethylpiperazine-*N*'-ethane sulfonic acid, pH 7.8; and 8% Ficoll 400), 1 μl Poly dIdC (4 mg/ml) (Sigma, St. Louis, MO), 0.2 μl $MgCl_2$ (100 mM), 0.2 μl dithiothreitol (10 mM), and 1 ng α-³²P–labeled double-stranded oligonucleotide containing the NF-κB DNA binding motif from the macrophage inflammatory protein-2 promoter (5'-CTCAGGGAATTTCCCTGG-3'). Sample mixtures were incubated at room temperature for 20 min, and protein DNA complexes resolved by 5% polyacrylamide gel electrophoresis under nondenaturing conditions. Gels were then dried, and autoradiograms developed and quantitated using a Bio-Rad PhosphorImager (Bio-Rad). To identify different proteins comprising the DNA–protein complex, supershift analysis was performed using antibodies recognizing p65 and p50 proteins (Santa Cruz Biotechnology, Santa Cruz, CA).

Transcriptional Activation of NF-κB–Dependent Gene Expression

C10 cells were stably transfected with the reporter plasmid 6KBTK-κB-luciferase (from Dr. Patrick Baeuerle, Tularik, Inc., San Francisco, CA), which contains three repeats of the HiV NF-κB sequences coupled to the luciferase gene (15). Transfected cells were allowed to grow to confluence in 10% FBS, then reduced to 0.5% FBS-containing medium 24 h before exposure to particulates. Total cell extracts were then prepared and assayed for luciferase activity (Luciferase Assay System; Promega Corp., Madison, WI), which was normalized to amounts of protein as described previously (14). Luciferase activity was expressed as total luciferase per microgram of protein. In experiments using antioxidants, stably transfected C10 cells were grown to confluence and then reduced to 0.5% FBS for 24 h before addition of $PM_{2.5}$ in HBSS or addition of $PM_{2.5}$ pretreated with the iron chelator deferoxamine mesylate (Sigma) at 1 mM in HBSS for 18 h. In other groups of cells, catalase (500 U/ml; Sigma) was added to medium for 1 h before addition of $PM_{2.5}$. These protocols have been used previously to prevent oxidant-induced activation of extracellular signal–regulated kinases by asbestos in mesothelial cells (17). After a 4-h exposure to particles, whole-cell extracts were prepared and assayed for luciferase activity.

Measurement of Oxidative Stress in Epithelial Cells

C10 cells at confluence were exposed to particles for 1, 2, 4, or 8 h. After the exposure, 2'-7'-dichlorofluoroscin diacetate (DCFDA) (10 μM; Sigma), the nonfluorescent precursor of fluorescein, was added to each dish at a final concentration of 10 nM for 30 min at 37°C. Cells then were trypsinized, washed, and resuspended in

HBSS without phenol red, and fluorescence was measured using a flow cytometer (Coulter Epics Elite; Coulter Corporation, Miami, FL) at excitation and emission wavelengths of 488 and 525 nm, respectively.

To determine the localization of oxidative stress in cells, C10 cells were grown to confluence and exposed to $PM_{2.5}$ (10 μg/cm²) or other particles for 1, 2, 4, or 8 h, and DCFDA was added for 30 min at 37°C. After cells were washed twice in HBSS without phenol red, they were observed using excitation from the 488-nM wavelength line of a krypton/argon laser on a confocal scanning laser microscope (Bio-Rad). Cells were viewed with a × 40 lens, Kalman fiber scanning (six scans), and setting at 30% laser intensity. Images were saved and then imported into the Confocal Assistant program. Control and treated dishes were scanned at the same setting parameters.

Statistical Analyses

All data were examined by analysis of variance using the Student–Newman–Keuls procedure to adjust for multiple pairwise comparisons between groups. For RPAs, two-way analysis of variance was used to test overall differences between sham and PM-exposed animals, averaged over both mouse species.

Results

Increased Expression of NF-κB–Responsive and Inducing Genes Are Observed in Mouse Lungs Exposed by Inhalation to PM

Figure 1 shows an autoradiogram (Figure 1A) and quantitation of results (Figure 1B) of groups of animal lungs at 24 h after a 6-h exposure to $PM_{2.5}$ using an RPA template for murine cytokine genes. Overall trend analysis indicated that significant increases ($P \leq 0.05$) in steady-state messenger RNA (mRNA) levels of TNF-α, TNF-β, IL-6, INF-γ, and TGF-β2 occurred in lung homogenates of $PM_{2.5}$-exposed mice, whereas mRNA levels of MIF and LT-β were unchanged in comparison with sham controls. No changes in gene expression were observed at the end of the 6-h exposure (data not shown). Data analysis indicated that trends of response to $PM_{2.5}$ by both species were similar. Mice ($n = 2$–3/

group/strain/time point) did not exhibit increases in total cell numbers nor increased proportions of neutrophils and/or lymphocytes in BALF at either time point (data not shown).

Increased NF-κB Binding to DNA and Transcriptional Activation of NF-κB Gene Expression Is Observed in Pulmonary Epithelial Cells after Exposure to PM

Figure 2 shows the time course of NF-κB binding to DNA in untreated C10 cells and those exposed to $PM_{2.5}$ (10 μg/cm²) over an 8-h period. The autoradiogram in Figure 2A shows the presence of the NF-κB subunit proteins p65 and p50 in the upper complex and p50 in the lower complex as identified by supershift analyses. There were no differences between complex composition in $PM_{2.5}$-exposed and sham untreated cells. Significant increases ($P \leq 0.05$) in DNA binding by NF-κB were observed in $PM_{2.5}$-exposed cells at 1 and 2 h, but not at later time periods (Figure 2B). To determine whether $PM_{2.5}$ and other particles caused increased transactivation of NF-κB, a time-course study was performed using a stable C10 NF-κB luciferase reporter cell line (Figure 3). These data showed that $PM_{2.5}$ caused significant ($P \leq 0.05$) increases in luciferase activity at 1 and 4 h. Elevations in activity by uCB were also observed at 2 and 4 h, whereas GB were inactive at all time points.

$PM_{2.5}$ and uCB Cause Intracellular Oxidant Production in Pulmonary Epithelial Cells

Figure 4 shows the quantitation of DCFDA oxidation, as measured by flow cytometry, in C10 cells exposed to particles (10 μg/cm²) at various time points over an 8-h period. At 1, 4, and 8 h, both $PM_{2.5}$ and uCB caused significant ($P \leq 0.05$) increases in fluorescence, whereas GB caused no changes in comparison with untreated control cells. Imaging using confocal scanning laser microscopy allowed us to visualize oxidation in epithelial cells over time in relationship to patterns of $PM_{2.5}$ particle distribution. As shown in Figure 5, oxidant localization in comparison with untreated cultures was strikingly increased in $PM_{2.5}$-exposed cells as
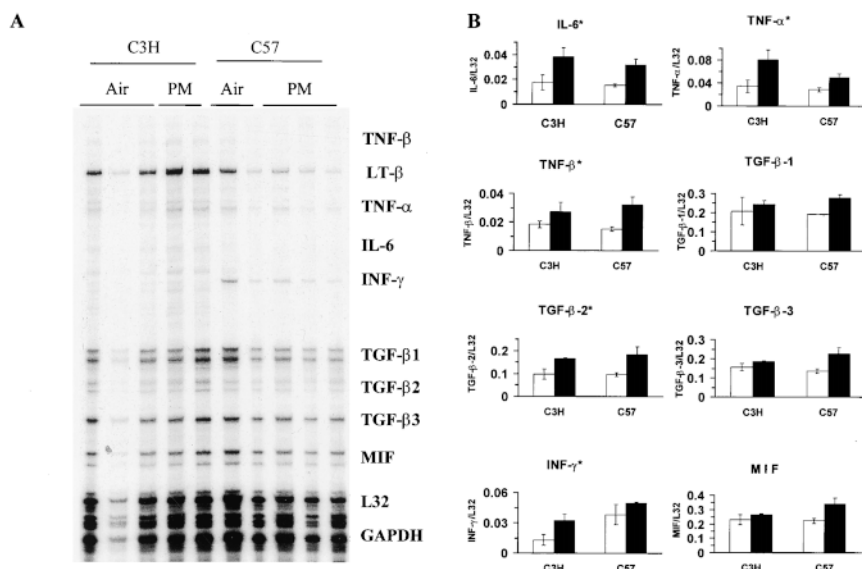

Figure 1. RPAs in lavaged lung tissues of C3H/HeJ and C57/BL6 mice exposed by inhalation (6 h plus a 24-h period in clean air) to NYC $PM_{2.5}$. (*A*) Autoradiogram developed to show relative amounts of mRNA for individual cytokines. (*B*) Quantitation of autoradiograms using PhosphorImaging. Sham = *open bars*; PM-exposed = *filled bars*. *Significant ($P \leq 0.05$) effect of PM exposure when differences from sham controls are averaged over both species.
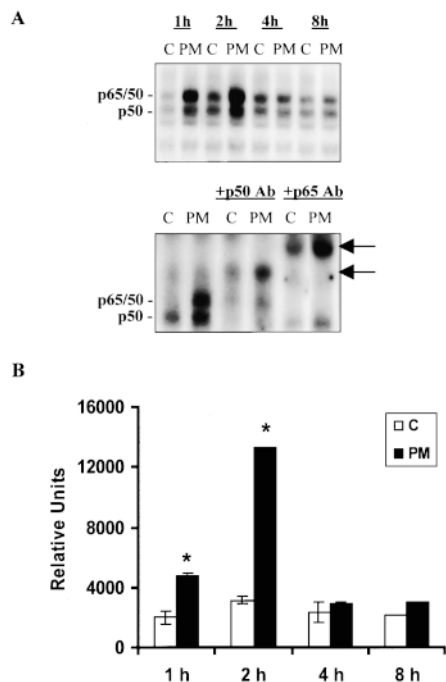
Figure 2. Time course of increased NF-κB binding to DNA as shown by electrophoretic mobility shift analysis in a pulmonary epithelial cell line (C10) exposed to PM$_{2.5}$. (*A*) Autoradiogram (*upper*) and supershift analysis at a higher magnification (*lower*). (*B*) Quantitation of results of experiments (*n* = 2) using PhosphorImaging. Intensity of bands is expressed in relative units. *$^*P \leq$ 0.05 in comparison with untreated control group at the same time period.

early as 1 h and appeared in cytoplasmic granules (Figure 5, *arrow*) consistent with the localization of particles. These increases in fluorescence were also observed with uCB but not GB (data not shown).

Transcriptional Activation of NF-κB–Dependent Gene Expression by PM$_{2.5}$ Is Prevented with Addition of Catalase

To determine whether oxidative stress was responsible for NF-κB transactivation by PM$_{2.5}$, we used established protocols (17), preadministering catalase for 1 h or pretreating PM$_{2.5}$ with the iron chelator deferoxamine, for 18 h
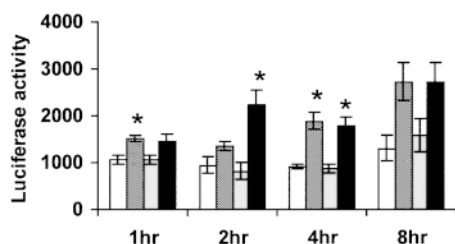

Figure 3. Transcriptional activation of NF-κB–dependent gene expression by VT PM$_{2.5}$ and uCB (10 μg/cm^2 dish) using a pulmonary epithelial cell line (C10) stably transfected with an NF-κB luciferase reporter plasmid. *Open bars*, control; *lightest grey bars*, glass beads; *darker grey bars*, particulate matter; *black bars*, ultrafine carbon black. *$^*P \leq$ 0.05 in comparison with untreated control group at the same time period.
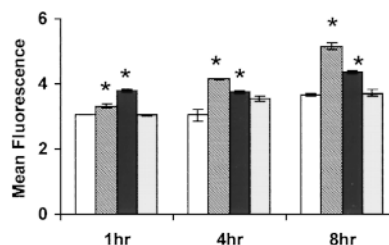

Figure 4. Oxidative stress in pulmonary epithelial cells as measured by DCFDA and flow cytometry. Note that protracted oxidant stress is increased significantly (*$^*P \leq$ 0.05) by VT PM$_{2.5}$ and uCB, but not by GB at an identical concentration (10 μg/cm^2 dish). *Open bars*, control; *lightest grey bars*, glass beads; *darker grey bars*, particulate matter, *black bars*, ultrafine carbon black.

before its addition to C10 cells stably transfected with a NF-κB luciferase reporter plasmid. Although pretreatment of PM$_{2.5}$ with deferoxamine was ineffective, addition of catalase significantly ($P \leq 0.05$) inhibited PM$_{2.5}$-induced NF-κB transactivation, indicating a possible role of hydrogen peroxide in the PM$_{2.5}$-associated response (Figure 6).

Discussion

Our studies indicate that PM$_{2.5}$ induces oxidant stress after uptake of particles by epithelial cells. This then causes increased translocation of the NF-κB subunits p50 and p65 to the nucleus and their increased binding to DNA. Consequently, transactivation of NF-κB–associated gene expression (i.e., TNF-α and IL-6) occurs. In addition, our *in vivo* studies showed elevated mRNA levels of these and other genes in lung that are associated with NF-κB activation in other cell types. Because cytokines regulated by or responsive to NF-κB may also activate NF-κB, this provides a positive feedback loop. Data in two strains of uncompromised mice showed that patterns of NF-κB–related cytokine gene expression in response to PM$_{2.5}$ were identical and occurred in the absence of obvious inflammation. However, because similar profiles of inflammatory proteins are increased in people with asthma and in patients with chronic obstructive and interstitial lung diseases (10, 18–20), exposures to PM$_{2.5}$ may also provide an amplifying loop that could explain the exacerbations of respiratory morbidity reported in these individuals. NF-κB appears to be chronically activated in macrophages and bronchial epithelial cells of people with asthma (21), thus providing another plausible mechanism for upregulation of these cytokines which may be further enhanced after PM$_{2.5}$ exposures.

Experiments using lavaged lung homogenates did not allow us to decipher precisely the cell types responsible for increased gene expression in response to PM$_{2.5}$. However, RPAs on C10 cells using the same cytokine template showed that steady-state mRNA levels of IL-6, a proinflammatory mediator, were significantly ($P \leq 0.05$) increased after exposures to PM$_{2.5}$ (10 μg/cm^2) for 2 h (data not shown). The earlier increases in mRNA levels of IL-6 in C10 cells may reflect the fact that particles directly contact epithelial cells within a 1-h period after their addition to cultures. Our results are consistent with increases in IL-6 expression observed in human bronchial epithelial cells *in*
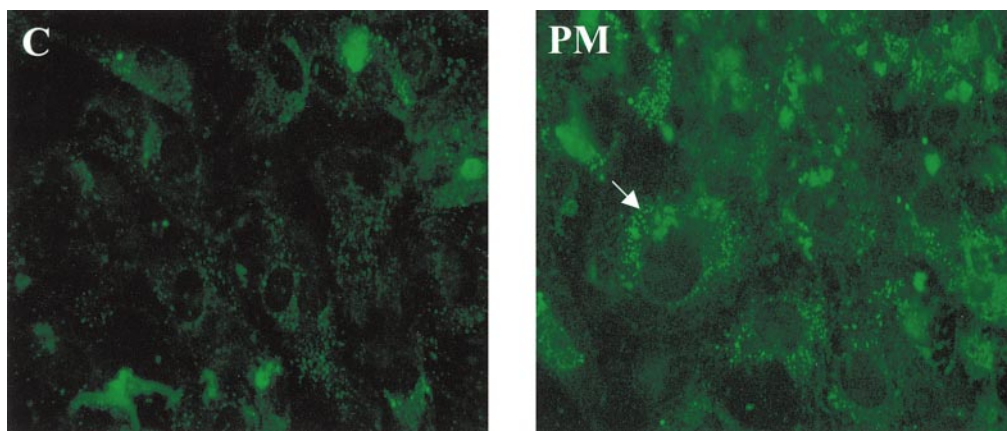
Figure 5. Imaging of oxidative stress in pulmonary epithelial cells exposed to VT PM$_{2.5}$ (10 μg/cm^2 dish for 1 h) as indicated using the DCFDA immunofluorescence technique and confocal scanning laser microscopy. Note increased perinuclear fluorescence (*arrow*) in PM-exposed cells in comparison with untreated control cells (C).

vitro in response to residual oil fly ash (ROFA), an emission source particle chemically and physically dissimilar from PM$_{2.5}$ (22). These studies, in concert, support the hypothesis that epithelial cells of the respiratory tract may be responsible *in vivo* for increased expression of some PM-induced cytokines in lung tissue. In addition, we have shown that increases in p65 protein, indicating NF-κB transactivation, occur selectively in rodent bronchiolar epithelium after brief inhalation of asbestos fibers (16).

The production of other cytokines induced by PM$_{2.5}$ in lung tissue may be associated with a number of cell types. TNF-α and TNF-β are proinflammatory cytokines with NF-κB binding sites in their promoter regions. The TNF-α gene is regulated by NF-κB and has been widely studied in a number of pulmonary and inflammatory diseases, where it appears to play a key role in the induction of oxidant stress and activation of NF-κB (reviewed in 10). TNF-α also can function as an activator of TGF-β (23), which also was increased in lungs after exposures to PM$_{2.5}$. Elevated mRNA levels of the potent immunomodulatory molecule IFN-γ were also noted in lung after exposures to PM$_{2.5}$.

Although IFN-γ does not activate NF-κB directly, it synergistically enhances TNF-α–induced NF-κB transactivation by a mechanism involving IκB degradation (24). Lung mRNA levels of MIF, a proinflammatory cytokine that has the unique potential to override the anti-inflammatory action of glucocorticoids (25), and LT-β, which binds to the LT-β receptor (i.e., a member of the TNF-receptor family), were unchanged after exposure to PM$_{2.5}$. It should be noted that patterns of expression of these cytokines, as well as increases in the cytokines described earlier in lung were identical in C3H/HeJ and C57/BL6 mice in the absence of increased inflammation in BALF. Although C57/BL6 exhibit greater inflammatory responses than do C3H/HeJ mice after acute exposures to ozone (11), inflammatory responses of these mice are comparative after infection with influenza virus, which is mediated in part by oxidant stresses (26). These studies, in concert, suggest that factors other than inflammation in BALF may determine strain sensitivity to oxidative stress.

Our studies here are the first to show increases in intracellular oxidant accumulation after exposure to PM$_{2.5}$ and establish a causal relationship between reactive oxygen species (ROS) and NF-κB activation by PM$_{2.5}$. First, we determined by flow cytometry and cell imaging techniques that oxidative stress was increased in cells exposed to PM$_{2.5}$ and uCB at time periods preceding maximum DNA binding and transactivation of NF-κB. A second approach was to pretreat cells with catalase before exposure to PM$_{2.5}$, a situation significantly ameliorating PM$_{2.5}$-induced NF-κB transactivation. These results implicate hydrogen peroxide as a mediator of NF-κB activity.

Although elemental static probe analysis of VT PM$_{2.5}$ particles revealed the presence of iron (14), and iron has been implicated in cell-free generation of the hydroxyl radical from PM (27) as well as increased ferritin synthesis (28), pretreatment of PM$_{2.5}$ particles with the metal chelator deferoxamine had no effect on reporter gene expression. Our results are in agreement with experiments demonstrating that TNF-α and IL-6 production by alveolar macrophages exposed to PM are not inhibited after pretreatment of PM with deferoxamine (29). These studies, in concert, indicate that iron-catalyzed generation of ROS may not be a predominant mechanism of PM$_{2.5}$-induced oxidant production in epithelial cells, a finding further
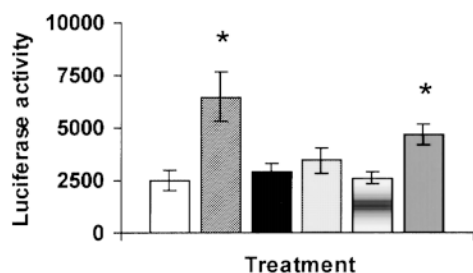

Figure 6. Prevention of transcriptional activation of NF-κB–dependent gene expression with addition of catalase (Cat) using a C10 epithelial cell line stably transfected with an NF-κB luciferase reporter plasmid. At confluence, cells were pretreated with various antioxidants before exposure to VT PM$_{2.5}$ (10 μg/cm^2 dish). *$P \leqslant$ 0.05 in comparison with no particle (untreated) group. *Open bar,* control; *very light grey bar,* Cat + PM; *second lightest grey bar,* PM; *darkest grey bar,* Def (deferoxamine) + PM; *dark-striped bar,* Def; *black bar,* Cat.

supported by our data showing increased and persistent oxidative stress induced by uCB, an iron-free component of PM. Because oxidant localization in PM-exposed C10 cells was associated with perinuclear accumulations of $PM_{2.5}$ consistent with phagolysosomes, it is conceivable that the respiratory burst associated with frustrated phagocytosis is a more relevant stimulus for oxidant production (30). Other studies examining NF-κB activation by ROFA also implicate other metals such as copper or vanadium (31, 32). Lipopolysaccharide (LPS) is a component of some urban PM samples and an inducer of NF-κB. However, LPS was not detected on filters after collection of New York City (NYC) PM.

Recently, we have also shown that $PM_{2.5}$ and ultrafine particles stimulate the *c-jun* kinase/stress-activated protein kinase cascade and levels of phosphorylated cJun immunoreactive protein (14). This pathway is also stimulated by oxidants in a number of cell types and is linked to transcriptional activation of activator protein-1, aberrant cell differentiation, and proliferation by pathogenic mineral dusts (33). These experiments and results here indicate that $PM_{2.5}$ and ultrafine particles can stimulate multiple signaling pathways leading to activation of transcription factors in pulmonary epithelium.

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